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Isolation and Identification of Resistant Microorganisms from Automotive Paint Sludge

Fatemeh Honarjooy Barkusaraey[1], Roya Mafigholami[2,*], Mohammad Faezi Ghasemi[3] and Gholam Khayati[4]

[1]Department of Environment, West Tehran Branch, Islamic Azad University, Tehran, Iran
[2]Department of Environmental Engineering, West Tehran Branch, Islamic Azad University, Tehran, Iran
[3]Department of Microbiology, Faculty of Basic Sciene, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[4]Department of Chemical Engineering, Faculty of Engineering, University of Guilan, Rasht, Iran

[*]*Corresponding author*: Department of Environmental Engineering, West Tehran Branch, Islamic Azad University, Tehran, Iran. Email: r.mafigholami@wtiau.ac.ir

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Abstract

Background: Paint coating systems are widely implemented on different surfaces for both aspects of decoration and protection against corrosion. Due to the presence of organic compounds, the growth of microorganisms is more likely to take place in paints, such as automotive paint. In the process of automotive painting, 20% - 60% of the paint does not expose to the automotive body, which is washed using water and would lead to the painting sludge formation. Paint sludge is considered one of the hazardous wastes from the automotive industry, which is finally landfilled or incinerated.
Objectives: Despite the presence of inhibiting compounds in paint sludge, such as heavy metals and biocides, the objective of this study was to isolate and identify microorganisms in the sludge culture.
Methods: The microorganisms were isolated using serial dilutions, direct cultivation, and enrichment methods in basic salt cultivation media. Then, their biochemical and molecular specifications were investigated.
Results: The number of microorganisms counted in paint sludge was approximately around 1×10^4 CFU/mL, and six isolated colonies were finally obtained.
Conclusions: The main isolated microbial consortium from paint sludge included *Pseudomonas aeruginosa*, *Staphylococcus haemolyticus*, *Micrococcus yunnanensis*, *Rothia amarae*, *Gordonia terrae*, and *Brevibacillus agri*. Nearly 83% of the isolated strains were Gram-positive.

Keywords: Paint Sludge, Automotive Industry, Cultivation Media, Six Bacterial Colonies, Resistant Microorganisms

1. Background

Paint is a synthetic material used as a substrate in the texture of furniture and other things (1). Paint is applied using a brush, roller, or spray as a thin layer on wood, metals, and stones to protect against corrosion (2) and create excellent decoration effect. The vehicle paint is a mixture of binders, pigments, solvents, and additives (3). Binders cause adhesion in paint particles, pigments create color and prevent corrosion, solvents lead to the dispersion of paint, and additives strengthen brushing and resistance properties (4). Automotive paint consists of a multilayer coating: primer as an anti-corrosion layer, a base coating that paints the automotive body, and a clear coating for a radiant appearance and UV protection (5). To produce the final automotive color, two types of paints are required: water-based and solvent-based (6). Methods for painting the automotive body include overspray, immersion, and powder methods (7). In overspray staining, only 50% - 80% of the paint reaches the automotive body (6), and 20% - 60% of the paint is transmitted as surplus (8) to the room with airflow and is successively removed using rotary washing water. The detergents added to the wash water separate the flocculants and coagulants from excess paint and contribute to the separation of paint from water (6). The mixture of water and spray paint, called paint sludge, is collected in a sludge pit (7).

Today, with the increasing number of vehicles around the world, the production of paint sludge is growing (6). Each factory produces about 10 - 15 tones sludge per day, containing some elements and contaminants (9). The combination of volatile organic compounds in paints can cause environmental pollution in the short or long-term (10). Ingredients such as antifouling Tributyltin (TBT) in

the paint are highly toxic to aquatic environments (2). Paint sludge is also a serious hazard to the health and environment, and in most cases, its production is inevitable (8). Eighty percent of environmental concerns in automotive painting factories are related to volatile organic compounds (VOCs), carbon dioxide (CO_2), and heavy metals. As a waste, paint sludge contains heavy metals and dissoluble organic carbon (DOC) of over 3,700 mg/L and, thus, is extremely hazardous. Paint sludge is classified in the EU code for waste disposal 080113 and is considered a waste with hazardous characteristics (6).

Salihoglu et al. investigated the possibility of composting automotive water-based paint sludge with sewage sludge of the same industry as substrate and corncob as a bulking agent in sex reactors.

Their results showed that carbon and nitrogen, organic matter and BTEX, nickel and tin, the ratio decreased (11). Ghomi Avili et al. (12) investigated chromium removal from automotive paint sludge using biological sludge and *Eisenia fetida* worms. The results showed that the Cr^{+6} concentration fell from 6 mg/kg to less than 0.2 mg/kg, pH decreased from 7.8 to 7.3, volatile solids decreased from 80.4% to 37%, and the C/N ratio decreased from about 27 to 14.3 after 90 days.

So far, various isolator microbial species have been isolated and identified from paint (13). Some organic and inorganic compounds are toxic to microorganisms. High quantities of heavy metals (14) and biocides in the paint can inhibit the metabolic activity of bacteria and fungi. Biocides can affect the growth of microorganisms through degradation of the cell membrane, inhibition of metabolic reactions, alteration of intracellular pH, and accumulation of toxic anions (15). Despite the presence of inhibitors in automotive paint sludge, the possibility of the isolation and identification of resistant microorganisms in the sludge was investigated in this study.

2. Objectives

Lots of research have been conducted on the isolation and identification of microorganisms from petroleum sludge, sewage sludge, contaminated coastal soils, mine soils, contaminated sediments, municipal waste, and wastewater sites for use in bioremediation. In the current study, the possibility of isolation and identification of resistant microorganisms in paint sludge was investigated.

3. Methods

3.1. Sampling

Paint sludge samples were collected from the site of an automotive painting factory under aseptic conditions using sterile spatula gathered inside 100 mL sterile Falcon tubes. The samples were then transferred to the laboratory at a temperature of 4°C.

3.2. Methods for Isolation of Microorganisms

3.2.1. Isolation by the Enrichment Method

Conventional salt-based media such as Bushnell Haas medium (BHMS) (16), mineral salt medium (MSM) (17), $9KFe^{2+}$ (18), 9Ks, and $9KNa_2S_2O_3$ (19) were used to isolate a wide range of bacteria. Culture media were prepared according to Table 1. Subsequently, 2% (w/v) of sterile paint sludge was added as a single source of carbon and energy. With some modifications in the amount of inoculation, 15 g of a paint sludge sample under aseptic conditions was added to the medium and incubated at 30 ± 2°C in a shaker incubator at 110 rpm for five consecutive days. To create colonies, 10 - 15 g of agar and 2% (w/v) sterile paint sludge were added to produce a solid medium. Then, 0.1 mL of each salt medium of Erlenmeyer cultured (triplicate of each medium) in nutrient agar (NA), potato-dextrose agar (PDA), and plate count agar (PCA) using the pour plate and steric methods and incubated at 30 ± 2°C for five days (20, 21).

3.2.2. Isolation by Direct Cultivation and Serial Dilution Method

Ten g of paint sludge sample was poured into a 250 cc Erlenmeyer under aseptic conditions, and 90 mL of sterile distilled water was added. The Erlenmeyer was covered with sterile cotton and homogenized with a shaker. Then, a portion of 1 mL was added to a tube containing 9 mL of sterile distilled water along with shaking. It continued serially to a dilution of 10 - 5. Then, 1 mL of each dilution and direct sample were cultured (triplicate of each dilution) on a plate containing NA and PDA. The plates were incubated at 30 ± 2°C for five days (22).

3.3. Morphological Properties

Gram-positive and Gram-negative strains were detected by Gram staining and microscopic observation.

3.4. Biochemical Properties

Species were isolated according to Bergey's manual of determinative bacteriology (23).

3.5. Molecular Identification

The 16SrRNA gene sequencing was performed by genomic extraction using Favorgen's Mini Kit Genetic DNA extraction kit. A polymerase chain reaction (PCR) was done to determine 16SrRNA gene proliferation using primers 4F: 5'-TATCGGAGAGTTTGATCCTGG-3' and 1541r: 5'-AAGGAGGGATCCAGCCGCA-3. The PCR program

Table 1. Composition of Salt Media for Isolation of Microorganisms

	Values
BHMS media	
$MgSO_4.H_2O$, g	0.2
$CaCl_2.H_2O$, g	0.002
KH_2PO_4, g	1
K_2HPO_4, g	1
NH_4NO_3, g	1
$FeCl_3$, g	0.05
Distilled water, cc	1000
pH	7
9KFe^{2+} media	
$(NH_4)_2SO_4$, g	3
KCL, g	0.1
K_2HPO_4, g	0.5
$MgSO_4.7H_2O$, g	0.5
$Ca(NO_3)_2$, g	0.01
Distilled water, cc	700
H_2SO_4 (w/v), N	10
$FeSO_4.7H_2O$, g	44.22
Distilled water, cc	300
pH	1 - 2
MSM media	
K_2HPO_4, g	0.05
$MgSO_4.H_2O$, g	0.5
$Ca(NO_3)_2$, g	0.01
KCl, g	0.1
$Na_2SO_4.10H_2O$, g	3.
$(NH_4)_2SO_4$	3
Distilled water, cc	1000
pH	7 ± 0.2
9KS media	
$(NH_4)_2SO_4$, g	3
KCl, g	0.1
K_2HPO_4, g	0.5
$MgSO_4.7H_2O$, g	0.5
$Ca(NO_3)_2$, g	0.01
Sulfur or thiosulfate, g	10
Distilled water, cc	1000
H_2SO_4, M	2
pH	6.5

was performed in 35 cycles (24), as given in Table 2. The PCR products were electrophoresed, and after observing the proper band, they were purified by Favorgen's Purification Mini kit. The DNA concentration after purification was measured at 260 nm. The purified PCR products were sent by the Iranian Research Organization for Science and Technology to Bioneer Company in Korea for sequencing. The sequencing was performed using primers as follows: 16r339: 5'-ACTGCTGCCTCCCGTAGGAG-3', 27f: 5'-GAGTTTGATCCTGGCTCAG-3', 704f: 5'-GTAGCGGTGAAATGCGTAGA-3'and 16f358: 5'-CTCCTACGGGAGGCAGCAG-3'. The obtained sequences were compared with nucleotide sequences available in valid databases, including NCBI and Eztaxon. Then, the phylogenetic trees were plotted using Mega version 6 software (25, 26).

Table 2. Polymerase Chain Reaction Program Protocol

Process	Temperature, °C	Time
Initial denaturation	95	3'
Denaturation	93	45"
Annealing	58	60"
Extension	72	90"

4. Results

The number of microorganisms counted with the plate count agar method was 1×10^4 CFU/mL. Six colonies were isolated on a saline culture medium, including BHMS, MSM, and 9K$Na_2S_2O_3$, and nutrient agar medium using direct and serial methods., using the direct and serial methods. For Gram staining, an expansion of isolated colonies was prepared, and the isolation of species was performed based on Bergey's manual. Some morphological and biochemical properties of the isolated microorganisms are given in Table 3.

To investigate the PCR products, electrophoresis was performed, and 1,500 nucleotide fragments were observed, according to Figure 1. The DNA concentration after purification at 260 nm was measured, and the results are presented in Table 4. The analysis disclosed sequences with different nucleotides. These sequences were compared by basic local alignment search tool (BLAST) with the nucleotide sequences available in valid databases, such as the National Center for Biotechnology Information (NCBI), and the results of the similarity percentage of the strains with the existing ones are presented in Table 5. Figure 2 indicates an example of a phylogenetic tree using Mega version 6 software.

Table 3. Morphological and Biochemical Properties of the Isolated Microorganisms

ID	aA	bB	cC	dD	eE	fF
Medium	BHMS	9K	MSM	NB serial	NB serial	NB direct
Gram stain	Gram-	Gram+	Gram+	Gram+	Gram+	Gram+
Morphology	Bacilli	Cocci	Cocci	Spherical	Coccoid	Rods
Colony shape	Circular	Circular	Circular	Circular	Rough	Flat
Colony color	Gray	White	Yellow	Cream	Orange	Gray
Size, mm	2 - 2.5	0.5 - 1	0.1.5 - 3	1	2 - 2.5	2 - 4
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	+
Coagulase	+	-	ND	ND	ND	ND
OF	Oxidative	Ferment	Inert	Ferment	ND	+
Motility	+	ND	ND	ND	ND	+
Indole	-	ND	ND	ND	ND	-
Urease	ND	+	-	-	+	-
Hemolysis	ND	ND	ND	ND	ND	Alfa
Nitrate reduce	ND	+	+	-	+	-
Starch	ND	ND	ND	ND	-	+
Lipase	+	ND	-	-	+	ND
Gelatin hydrolysis	+	ND	ND	ND	ND	ND
Denitrification	+	ND	ND	ND	ND	ND
Hemolysis	ND	+	ND	ND	ND	ND
ONPG	ND	-	ND	ND	ND	ND
MR/VP	ND	ND	-/-	ND	ND	ND/-

Table 4. The Concentration of Purified DNA

Strain Code	DNA Concentration, ng/μL
Strain aA	154
Strain bB	87.5
Strain cC	89.6
Strain dD	70.7
Strain eE	93.8

5. Discussion

The present study showed that each salt medium provided growth conditions for some of the microorganisms found in paint sludge. *Micrococcus yunnanensis* was isolated from paint sludge in MSM, while in the same medium, Phulpoto et al. (27) isolated *Brevibacillus parabrevis* strains from oil-based paint sludge and Ashwini et al. (28) isolated *Pseudomonas*, *Staphylococcus*, and *Lactobacillus* stains. In the direct and serial culture of paint sludge on the NA medium, bacteria, including *Rothia amarae*, *Gordonia terrae*, and *Brevibacillus agri* were isolated. Obidi et al. (29) could successfully isolate *Bacillus brevis*, *B. laterosporus*, *B. polymyxa*, *Escherichia coli*, *Lactobacillus gasser*, *Lactobacillus brevis*, and *Proteus mirabilis* from fresh industrial paint on the NA medium and *Aspergillus* on the PDA medium. In the current study, no fungal colonies and *Acidithiobacillus* strains were isolated on PDA and 9k medium, respectively.

The Bushnell Haas medium that was applied to cultivate hydrocarbon-degrading microorganisms showed the most suitable growth of the species. Further, only *Pseudomonas* bacteria grew on the Bushnell Haas medium. The microbial population of paint sludge was counted as 1×10^4 CFU/mL on the PCA medium, whereas Maduka et al. (30) isolated microorganisms between 0 and 5.4×10^5 CFU/mL from emulsion paints and between 0 and 6.5×10^5 CFU/mL from clear paints. The isolated species were mostly pathogens that were isolated from clinical samples and soils, but the strains were different from previous studies. The bacteria *Micrococcus yunnanensis*, *Staphylococcus haemolyticus*, and *Pseudomonas aeruginosa* were isolated from the paint sludge. The sequences compared to the

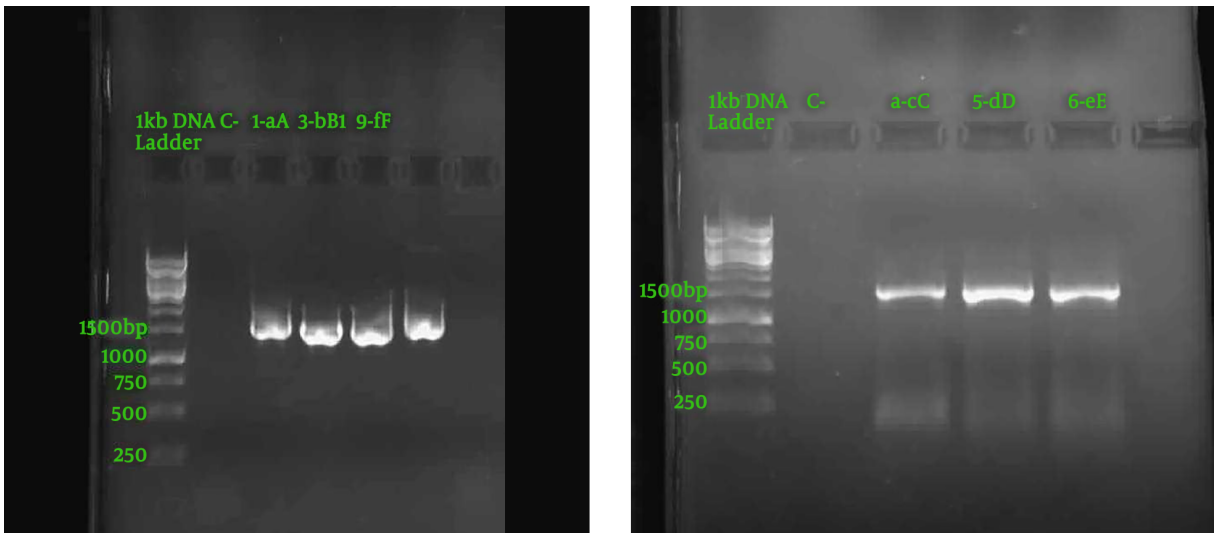

Figure 1. PCR product electrophoresis

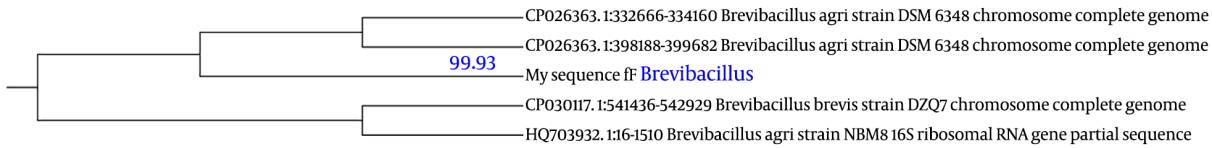

Figure 2. The phylogenetic tree of the fF-Brevibacillus strain plotted with Mega software version 6.

Table 5. The Comparison of the Similarity Percentage of Strains with National Center for Biotechnology Information

Strain Code	16s rRNA Sequence	Identification	Similarity, %
Strain aA	1496 nucleotides	*Pseudomonas aeruginosa* JCM 5962(T)	100
Strain bB	1516 nucleotides	*Staphylococcus haemolyticus* MTCC 3383(T)	99.86
Strain cC	1484 nucleotides	*Micrococcus yunnanensis* YIM 65004(T)	99.86
Strain dD	1492 nucleotides	*Rothia amarae* JCM 11375(T)	99.79
Strain eE	1483 nucleotides	*Gordonia terrae* NBRC 100016(T)	100
Strain fF	1495 nucleotides	*Brevibacillus agri* DSM 6348(T)	99.93

NCBI were 99.86% and 100% similar to YIM 65004, MTCC 3383, and JCM 5962 type strains, respectively. Opperman and Goll (31) isolated *Bacillus*, *Pseudomonas*, *Proteus*, *Serratia*, and *Enterobacter* as Gram-negative bacteria and *Micrococcus* and *Clostridium* as Gram-positive strains from water-based paints. Cappitelli et al. (32) isolated *Staphylococcus* from surfaces coated with epoxy resins, which was examined in terms of bioremediation. *Brevibacillus agri* isolated from the paint sludge had a 99.93% similarity to the DSM strain. Phulpoto et al. (27) in a study on paint bioremediation could isolate *Brevibacillus parabrevis* strain NAP3 from solvent-based paint. *Rothia amarae* strain was isolated from the paint sludge with 99.79% similarity to JCM 11375 strain but this bacterium was isolated from wastewater sludge by Fan et al. (33). In this study, *Gordonia terrae* strain with 100% similar to NBRC 100016 strain isolated from paint sludge. Recently, many *Gordonia* strains have isolated from various medium sources, such as soil, some of which were involved in the degradation of xenobiotic compounds, such as benzopyridine (34).

5.1. Conclusions

According to strains obtained in this study and the presence of resistant microorganisms in the automotive paint sludge, one can investigate the idea of the bioremediation using microorganisms existing in this type of sludge to reduce the pollution load and make the compliance with landfill standards.

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Footnotes

Authors' Contribution: Fatemeh Honarjooy Barkusaraey performed the experiments. Fatemeh Honarjooy Barkusaraey, Roya Mafigholami, Mohammad Faezi Ghasemi, and Gholam Khayati performed the literature review, data collection, analyzed and interpreted the data, and prepared the manuscript text.

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